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**FLUORESCENCE IMMUNOFILTRATION
ASSAY OF BRUCELLA MELITENSIS (U)**

BY

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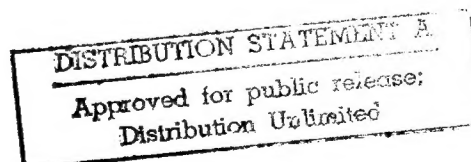
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William E. Lee, John G. Hall and H. Gail Thompson

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ABSTRACT

Detection of biological materials can be carried out using a silicon-based light addressable potentiometric (LAP) sensor in conjunction with filtration-capture immunoassay. The immunoassay employs a fluorescein-conjugated antibody directed against a target antigen plus a second urease-labelled antibody directed against fluorescein. The assay system is useful for measuring protein, virus and bacteria in aqueous samples and has been employed in automated prototypes of the Biochemical Detector. Although fluorescein is employed in the assay as a binding site for the signal-generating urease-labelled antibody, it is a highly fluorescent molecule and has signal-generating capacity of its own. In the present work a comparison was made of the sensitivity of detecting filtration-captured bacteria, *Brucella melitensis*, via a LAP sensor assay and via fluorescence derived from fluorescein-labelled antibodies. Limits of detection for *Brucella melitensis* were 0.5 ng per well for the LAP sensor and 10 ng per well for the fluorescence detection. Although the fluorescence system was not as sensitive as the potentiometric assay, the results are none the less encouraging since the optical configuration of the fluorescence assay was not optimized. The fluorescence-based assay offers a number advantages over the potentiometric sensor for an automated Biochemical Detector, including speed of measurement, greater stability of reagents and absence of substrate fluidics.

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INTRODUCTION

A detection technology based on a light addressable potentiometric (LAP) sensor (1) has been successfully employed to detect the presence of protein, virus and bacteria (2, 3, 4, 5) in aqueous samples. It has also been employed in the Biochemical Detector, a biosensor detection system for the rapid assay of biological material. For the detection of a particular biological analyte, antibodies directed against the analyte (antigen) are reacted in a homogeneous liquid phase with the antigen. The resulting immune complexes are filtered through nitrocellulose membranes and are immobilized on a membrane either through biotin-streptavidin interaction (6) or through passive capture (in the case of bacteria) as depicted by Figure 1. A second antibody conjugated to an enzyme (urease) is subsequently filtered through the membrane and a detectable immobilized immune complex is obtained by attachment of the second antibody (Fig. 1a). The presence of antigen is determined by monitoring the membrane for urease activity by means of the pH-sensitive LAP sensor. In the LAP sensor assay the use of fluorescein/anti-fluorescein binding pairs permits a generic signal-producing antibody (urease-labelled) to be employed in all assays, independent of the specific antigen.

Although fluorescein is used as an antigenic target (that is, for its capacity to bind strongly to antibody) in the LAP sensor system, it does possess specific spectral properties that can be used in immunoassay detection (7). Fluorescein strongly absorbs visible light in the range of 490-510 nm, with a coefficient of extinction of about $60,000 \text{ M}^{-1} \text{ cm}^{-1}$, and fluoresces at somewhat longer wavelength with an efficiency that is close to 1.0 (8). Thus an immobilized immune complex, as depicted in Fig. 1b is potentially capable of being detected by fluorescence. In this scheme, the second antibody, anti-fluorescein urease conjugate, is not required.

LAP sensor assays employing passive capture of bacteria have been developed for *Brucella* and other bacteria (5, 9). However detection of immobilized immune complex on membrane by means of the fluorescence emission from labelled antibodies has not been previously undertaken. In order to study this emission the present work was carried out. The purpose was to compare the detection methods using a common antigen, *Brucella melitensis*, and common experimental conditions and to determine the limits of detection of the fluorescence assay and the sensitivity relative to the LAP sensor.

EXPERIMENTAL SECTION

Materials

Bovine serum albumin (BSA), phosphate buffered saline (PBS), Tween 20, Triton X-100 and urea were obtained from Sigma Chemical Co. (St Louis, MO) and used without further purification. Anti-fluorescein urease conjugate was obtained from Molecular Devices Corp. (Menlo Park, CA) as a lyophilized powder. A sample vial was reconstituted in 10 mL distilled water. Formalin killed lyophilized *Brucella melitensis* was obtained from Dr John Cherwonogrodsky (DRES) and reconstituted in PBS, at 20 mg/mL. Polyclonal (rabbit) antibody directed against *Brucella melitensis* was obtained from the Molecular Biology Group (DRES) and was labelled with fluorescein using a hapten labelling kit (Molecular Devices Corp.). The antibody concentration was 0.34 mg/mL and the molar incorporation ratio of fluorescein was 5.0.

Wash buffer consisted of PBS, pH 6.5, plus 0.2% Tween 20 detergent. The assay buffer was the wash buffer titrated to pH 7.0, containing 1% albumin and 0.25% Triton X-100. The substrate solution for the enzyme assays was wash buffer containing 100 mM urea.

Apparatus

A commercially available LAP sensor, marketed under the name Threshold Unit™ was purchased from the manufacturer, Molecular Devices Corp. The instrument was controlled by an IBM PS/2 model 30 microcomputer and custom designed software supplied by Molecular Devices Corp. The design of the Threshold Unit allowed eight samples to be tested simultaneously. Nitrocellulose membrane filters (0.44 μ m pore size) coated with BSA were purchased from Molecular Devices Corp. A Titertek Fluoroscan II fluorescence microtiter plate reader was obtained from Flow Laboratories/ICN (Montreal, PQ) and was controlled by a 286 microcomputer and Automate (version 2.2) software (ICN). Black polystyrene 96 well microtiter plates were obtained from Dynatek Laboratories (Chantilly, VA)

Immunoassay Procedures

Immunoassays were carried out according to the reaction scheme shown in Figure 1. An antibody reagent solution consisted of 150 μ L of stock fluorescein-labelled anti-Brucella in 15 mL of assay buffer. The stock Brucella antigen was diluted in assay buffer so that the required amounts for calibrators and unknowns were contained in 100 μ L. An aliquot of 100 μ L diluted Brucella was mixed with 100 μ L of antibody reagent solution, incubated for 5 min

at room temperature, and filtered at $130 \mu\text{Lmin}^{-1}$ through nitrocellulose membrane on the filter apparatus of the Threshold work station. Each well on the membrane was rinsed with $500 \mu\text{L}$ of wash buffer. The membrane, which contained the immobilized immune complex, could be used either for LAP sensor or fluorescence detection.

For the LAP sensor assays $100 \mu\text{L}$ of reconstituted anti-fluorescein urease conjugate was filtered through each well at $65 \mu\text{Lmin}^{-1}$. The membrane was rinsed with wash buffer, $500 \mu\text{L}$ per well, and inserted into the LAP sensor reader compartment. The enzyme activity associated with each well was recorded as $\mu\text{V s}^{-1}$.

For the fluorescence assays the membranes containing the immobilized complexes were removed from the filter apparatus and allowed to air dry. Circular sections (radius 3 mm) containing the immobilized immune complex were punch-cut out and placed in the wells of microtitre plates. The plate was inserted into the plate reader and the fluorescence was recorded as relative intensity units.

RESULTS

Fluorescence Response of *Brucella melitensis*

A standard curve of the fluorescence response of *Brucella melitensis* is shown in Figure 2. The standards, ranging from 300 to 5000 ng per well, gave a monotonic increase in fluorescence signal, although at higher concentrations the overall sensitivity (as measured by the slope, $\Delta\text{signal}/\Delta\text{ng}$) decreased. For comparison a LAP sensor assay of *Brucella melitensis* is shown in Figure 3. The concentration range of standards shown for the LAP sensor (3-50 ng) is less than that for the fluorescence assay. However, each curve

demonstrates the useful range. The LAP sensor assay was optimized for high sensitivity (i.e., low analyte concentration) and as such there was an upper limit of about 100 ng per well at which the signals were too large to effectively measure (data not shown).

Effects of Membrane Hydration

The emission of immune complex immobilized on nitrocellulose membrane was dependent upon the degree of hydration. Standard curves of Brucella with dry, moist and wet membrane are shown in Figure 4. The range of analyte concentration shown is similar to concentration range given for the LAP sensor assay (Fig. 2). Both the dose response (slope of the plots in Fig. 4) and the background (zero antigen) decreased with increasing hydration. However in terms of a signal-to-noise ratio (S/N), whereby the signal was taken to be the slope and the noise was taken to be the background of the experiment (reagents but no antigen), the wet membranes yielded the greatest S/N (see caption Fig. 4). The overall decrease in signal levels with hydration is primarily due to the decrease in the scattering of light from the surface of the membrane. The membrane itself is not a smooth continuous surface, rather a fibre matrix with pores of approximate nominal size, $0.44\ \mu\text{m}$ and a large surface area for scattering of light (10). Scattered light can originate from a number of sources including elastic scattering, Raman scattering, and refractive index change and is particularly detrimental to fluorescence measurements (11). The scattering of light combined with multiple reflections from the membrane matrix also magnifies any inherent fluorescence emission of the membrane itself. The presence of water on the membrane has the effect of providing a more uniform refractive index over the irradiated area by coating the matrix and filling the interstitial volume with liquid. The refractive index of nitrocellulose polymer (1.45) is more closely matched to water (1.33) than to air (1.00) (12). In the

present study, fluorescence measurements were made using fixed wavelengths for excitation and emission (480 and 520 nm, respectively) and light scattering was observed. However, since the spectral dependence of overall emission (fluorescence plus scatter) could not be measured, it was not possible to determine the specific source or nature of the scattered light.

Detection of *Brucella Melitensis*

As observed in previous work detailing filtration-capture immunoassays (4,5), longer incubation times in general provided higher signals and increased sensitivities as a consequence of the greater number of immune complexes formed. An indicator of sensitivity is the lower limit of detection (LOD), which in this work was taken to be the mass of antigen which produced a signal equal to the background of the assay plus two standard deviations. The LODs determined from wet filters were 33 and 10 ng per well, respectively, for 5 and 60 min incubations and somewhat higher for dry filters. The results are summarized in Table I. A comparable assay for *Brucella melitensis* employing passive filtration capture and LAP sensor detection was found to have a LOD of 0.5 ng per well for 60 min incubations.

Quantitations of unknown samples of *Brucella* were carried out by fluorescence and LAP sensor detection and the results are given in Table II. Linear regression analysis of calculated versus actual amounts yielded r^2 values of 0.996 and 0.999 for fluorescence and LAP sensor data, respectively.

DISCUSSION

The fluorescence detection of bacteria immobilized on nitrocellulose has been demonstrated and a comparison made with the sensing techniques used in the Biochemical Detector, namely, a light addressable potentiometric sensor. The limits of detection for the fluorescence assay were higher than that for the LAP sensor, 10 ng per well versus 0.5 ng per well for a 60 min incubation assay. On the other hand, the fluorescence assay was responsive at higher concentrations of analyte where the LAP sensor was effectively off-scale.

Although the sensitivity of the fluorescence assay was not as great as that of the LAP sensor, it has the capacity to be enhanced. The assay employed a fluorescence microtitre plate reader, designed for measuring emission of liquids in plastic wells, rather than a specifically constructed sensing device. The optical configuration was not optimized to read nitrocellulose membranes. Thus, with the microplate reader there was high background scatter observed from the nitrocellulose surface. Overall, the system can be improved in a number of ways. The spectroscopic label, in present case fluorescein, is a highly fluorescent molecule but nonetheless could be replaced. Two likely choices would be chelated lanthanide group metal ions or phycobiliprotein.

The former are used in time resolved fluorescence immunoassay (13), an emerging detection technique whereby certain metal ions (e.g. europium) are chelated to antibodies. Emission of light from the chelated ions is relatively long-lived (microseconds) compared to fluorescence from organic compounds (nanoseconds) or to scattered light. Therefore, interfering emission from other sources decays away prior to the measurement of the chelated ion emission. The latter, phycobiliproteins, are a class of stable and highly soluble fluorescent proteins derived from cyanobacteria and eukaryotic algae and are involved in

the collection of light for photosynthesis. Because of their unique role, phycobiliproteins have evolved to maximize both absorbance and fluorescence and to minimize quenching, derived from either internal pathways or external factors, such as pH or ionic composition. They have exceptionally large absorbance coefficients (as high as $2.4 \times 10^6 \text{ M}^{-1}\text{cm}^{-1}$) and quantum efficiencies, up to 0.98 (14) and are employed in fluorescence activated cell sorting (15) and immunoassay (16, 17).

Electron microscopy has shown nitrocellulose membrane to have a highly irregular matrix structure (10). For $0.44 \mu\text{m}$ grade membrane the pore sizes are of the same order in magnitude to the wavelength of the light used (500 nm). Thus the rather large background signals observed in the experiments were most likely due to scattered light from the rough membrane surface, although some inherent fluorescence of the membrane material itself cannot be discounted as a source of background. When the membrane was wetted with distilled water, thereby filling the interstitial volumes and providing a smoother more continuous surface, the background (fluorescence) signal decreased to about 20% of that recorded on dry membrane. Along with the decrease in background was an increase in the signal-to-background ratio. Other methods of improving the signal are available. The use of fluorophores with larger Stokes shifts than fluorescein would permit a larger spectral separation between the excitation and emission radiation bands and potentially offer lower background. The use of polarized light would also diminish the amount of scattered light in the emission. This arises from the fact that fluorescence emission is generally polarized parallel to the excitation, whereas scattered light tends to be depolarized. Materials other than nitrocellulose may provide smoother surfaces. Teflon has recently been demonstrated to be useful solid support in bioassay systems (18). Figure 5 provides a schematic representation of a fibre optic detector suitable for measuring emission from fluorophores immobilized on a membrane surface.

The measurement of visible radiation is a well understood process and relatively easy to accomplish. Photodetectors that are highly sensitive, compact and rugged can be readily fabricated. Since photons will pass readily through a liquid-air interface, no physical contact between the assay surface (nitrocellulose membrane) and the detector is required. A spatial separation of this type is not feasible in the LAP sensor and build-up of biological debris on the sensing elements, which causes interference, has been observed with highly concentrated proteinaceous samples (9). This is a problem that has not yet been overcome on the BCD.

The work described herein presents an alternative sensing technique for rapid immunofiltration assays, of the type employed in the BCD. This method possesses a number of advantages over the current BCD system. The assay does not require an enzyme for signal generation. This removes one inherently unstable component from the assay and simplifies the fluidics design by eliminating the need for a second antibody. In addition, the need for substrate solution, which in the current design of the BCD is the largest consumable liquid, is also eliminated. Photonic measurements can be carried out more rapidly than the potentiometric measurements and the detector sensing times can be reduced from approximately one minute to several seconds. Since physical contact between the assay surface and the detector is not required, a greater maintenance-free operational period can be expected. At present, access to the current BCD sensor technology, the LAP sensor, is encumbered by patents. This would not be the case with a fluorescence-based sensor.

CONCLUSIONS

This work is the first to report a rapid immunofiltration assay using fluorescence-based detection. The study has demonstrated an alternative sensing technique for the Biochemical Detector. The system described offers a number of potential advantages over the current technology, including speed of measurement, simplicity of design, stability of reagents and absence of patent encumbrances.

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Table I. Limits Detection of *Brucella melitensis*

Assay	LOD (ng per well)	
	5 min ⁺	60 min ⁺
Fluor (wet) [*]	33	10
Fluor (dry) [*]	45	24
LAP sensor	2	0.5

⁺incubation time^{*}fluorescence assay on wet or dry membrane

Table II. Quantitation of *Brucella melitensis*

Actual Amount (ng)	Measured Amount (ng)	
	Fluorescence	LAP sensor [*]
3000	3440 (280)	3210 (55)
2000	2468 (230)	2210 (48)
1000	1281 (130)	1040 (42)
500	524 (13)	570 (40)

^{*}assayed at 100X dilution

the numbers in brackets following the calcd. values are standard deviations, n=4

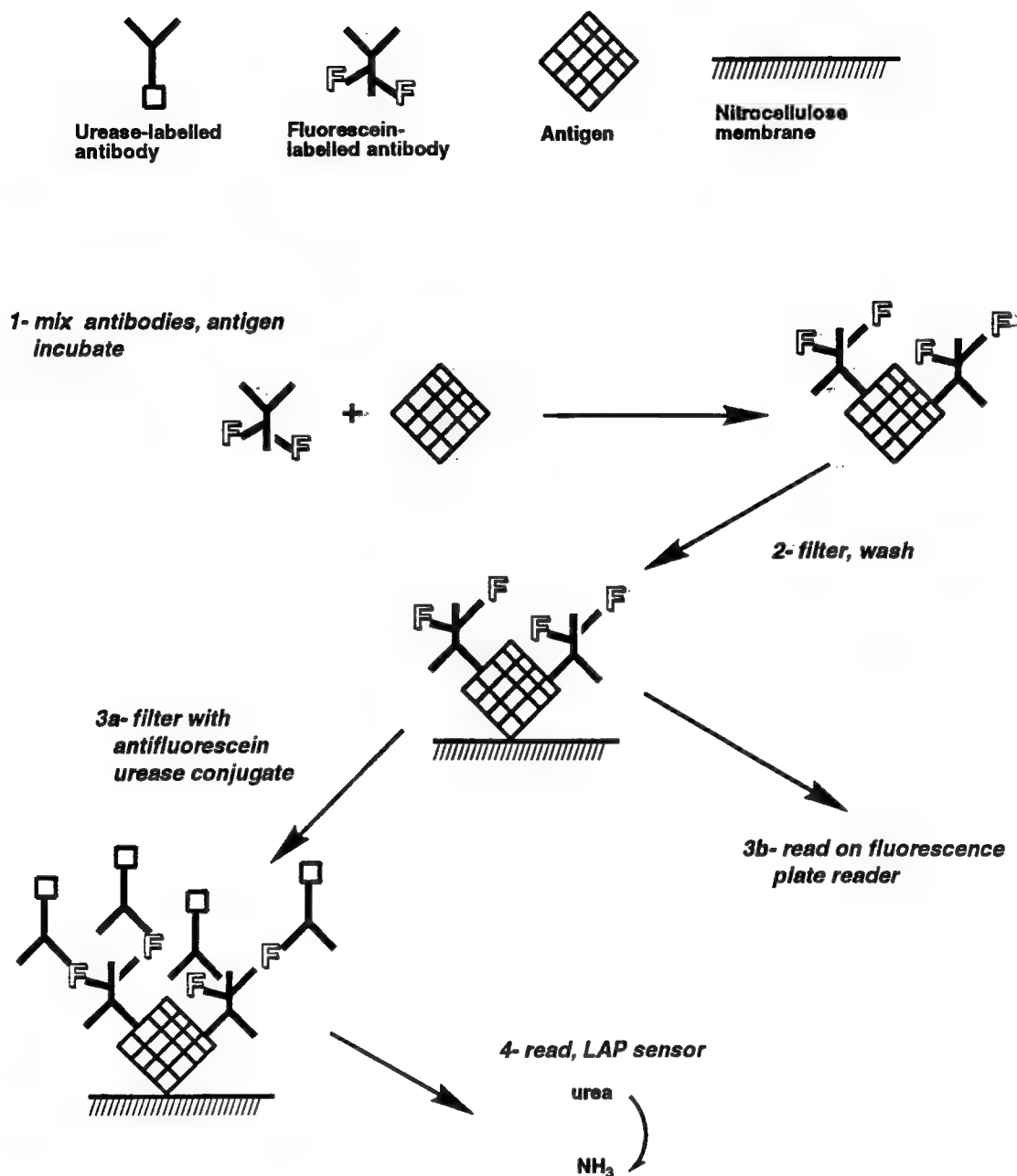


Figure 1. The reaction scheme for *Brucella melitensis* immunoassays. Steps 1 & 2 are common to both LAP sensor and fluorescence detection. In the LAP sensor assay (a), an anti-fluorescein urease conjugate is added to the immobilized antibody complex and the membrane is monitored for urease activity. In the fluorescence assay (b), the fluorescein labels are irradiated with visible light (480 nm) and the emission is monitored at 520 nm.

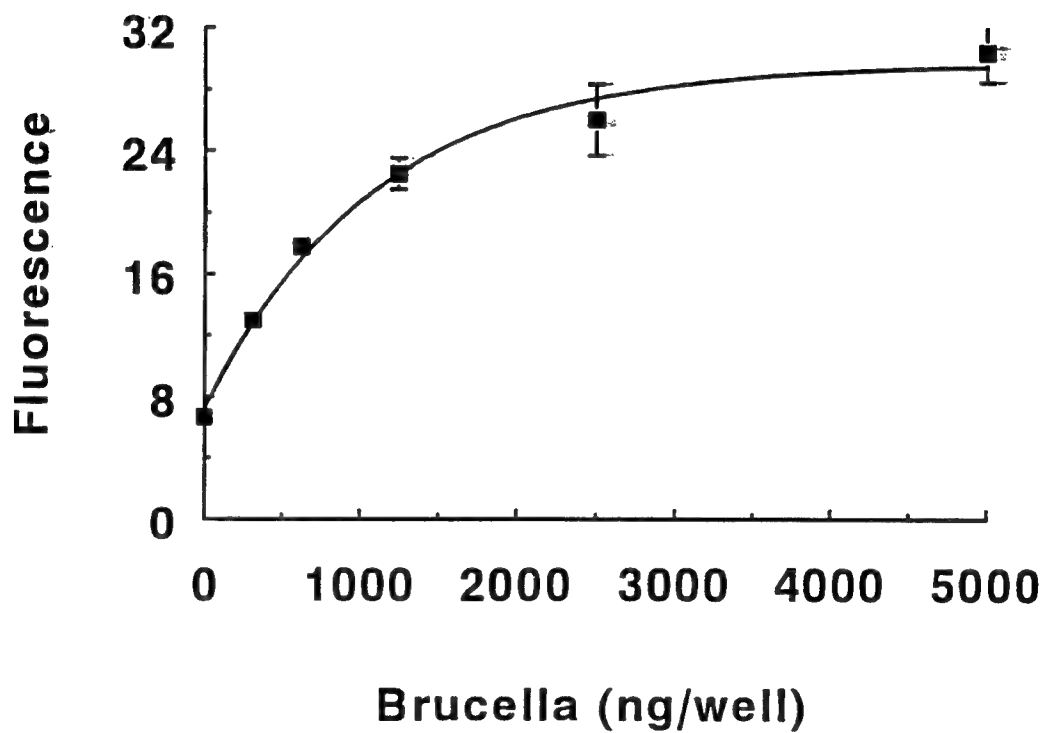


Figure 2. A standard curve of *Brucella melitensis* determined from the emission of fluorescein-labelled antibodies, according to the reaction scheme in Figure 1. The fluorescence intensity (y-axis) is given in arbitrary units.

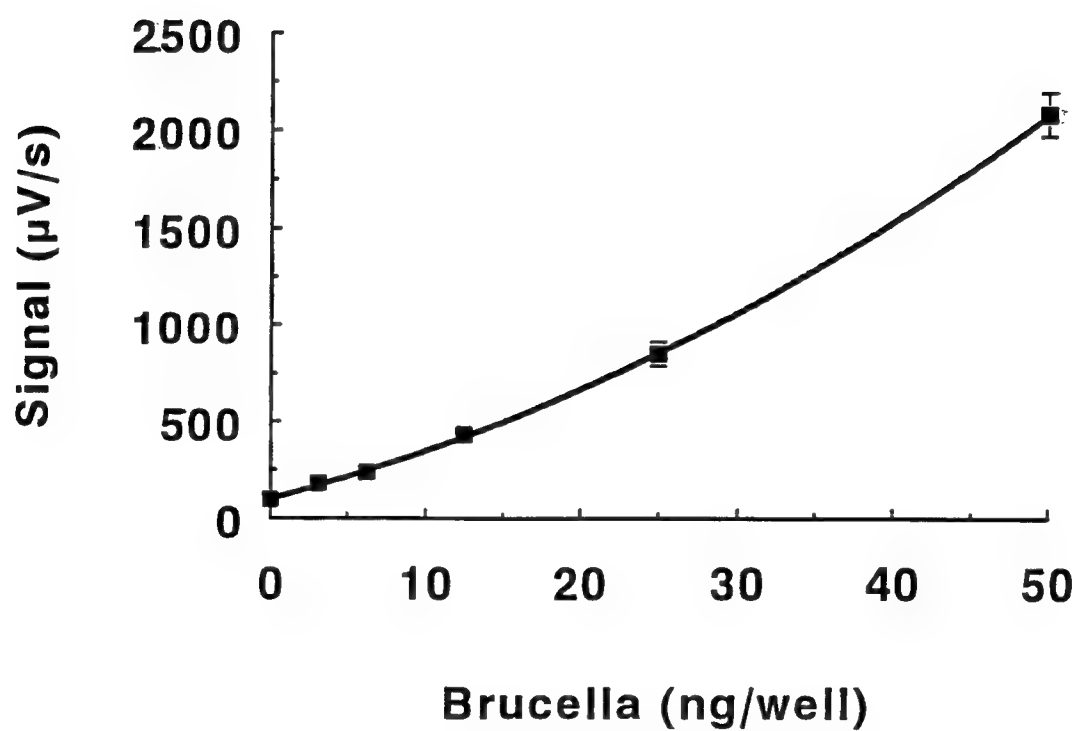


Figure 3. A standard curve of *Brucella melitensis* determined by LAP sensor detection, according to the reaction scheme shown in Figure 1. The standards depicted above were those used in Figure 2, diluted by 100.

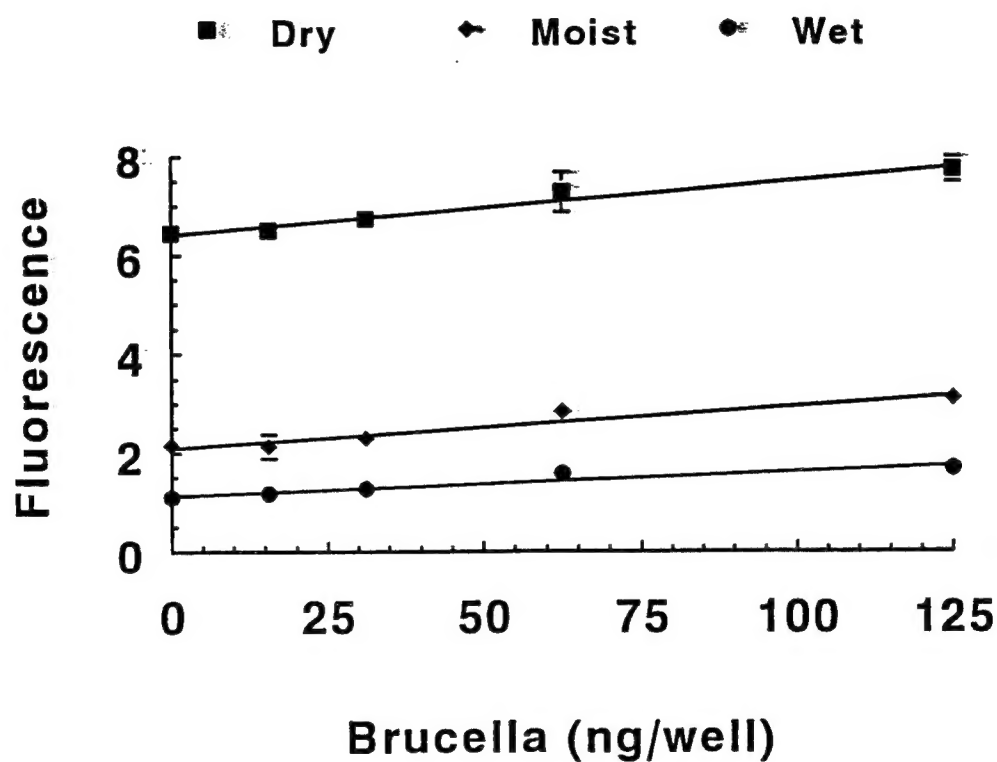


Figure 4. The effects of hydration on the background and dose response of a fluorescence *Brucella melitensis* assay. For dry, moist and wet membranes the backgrounds were 6.65, 2.15 and 1.10 arbitrary units (a.u.), respectively; the slopes were 0.0112, 0.0089 and 0.0046 a.u./ng, respectively. The ratios of slope to background (S/N) were 1.7 , 4.1 and 4.6×10^{-3} , respectively. The fluorescence intensity (y-axis) is given in arbitrary units

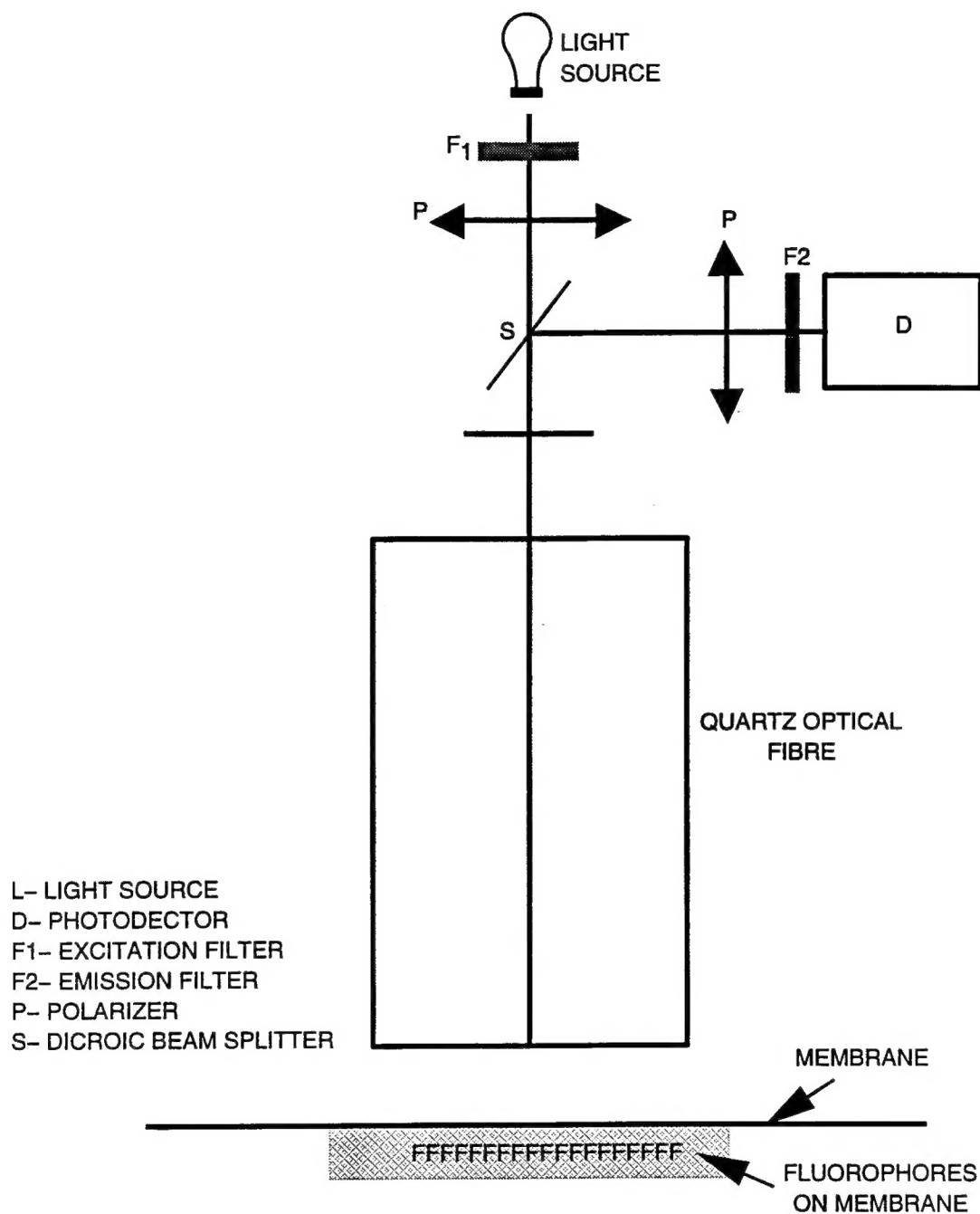


Figure 5. The layout of a fibre optic sensor device for measuring fluorescence from membranes. L, light source; D, photodetector; F₁, excitation filter; F₂, emission filter; P, polarizer; S, dichroic beam splitter.

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3. TITLE (the complete document title as indicated on the title page. Its classification should be indicated by the appropriate abbreviation (S,C,R or U) in parentheses after the title.) Fluorescence Immunofiltration Assay of Brucella Melitensis		
4. AUTHORS (Last name, first name, middle initial. If military, show rank, e.g. Doe, Maj. John E.) William E. Lee, John G. Hall and H. Gail Thompson		
5. DATE OF PUBLICATION (month and year of publication of document) January 1995	6a. NO. OF PAGES (total containing information. Include Annexes, Appendices, etc.) 20	6b. NO. OF REFS (total cited in document) 18
6. DESCRIPTIVE NOTES (the category of the document, e.g. technical report, technical note or memorandum. If appropriate, enter the type of report, e.g. interim, progress, summary, annual or final. Give the inclusive dates when a specific reporting period is covered.) Suffield Memorandum 1407		
8. SPONSORING ACTIVITY (the name of the department project office or laboratory sponsoring the research and development. Include the address.) Defence Research Establishment Suffield PO Box 4000 Medicine Hat, AB T1A 8K6		
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Detection of biological materials can be carried out using a silicon-based light addressable potentiometric (LAP) sensor in conjunction with filtration-capture immunoassay. The immunoassay employs a fluorescein-conjugated antibody directed against a target antigen plus a second urease-labelled antibody directed against fluorescein. The assay system is useful for measuring protein, virus and bacteria in aqueous samples and has been employed in automated prototypes of the Biochemical Detector. Although fluorescein is employed in the assay as a binding site for the signal-generating urease-labelled antibody, it is a highly fluorescent molecule and has signal-generating capacity of its own. In the present work a comparison was made of the sensitivity of detecting filtration-captured bacteria, *Brucella melitensis*, via a LAP sensor assay and via fluorescence derived from fluorescein-labelled antibodies. Limits of detection for *Brucella melitensis* were 0.5 ng per well for the LAP sensor and 10 ng per well for the fluorescence detection. Although the fluorescence system was not as sensitive as the potentiometric assay, the results are nonetheless encouraging since the optical configuration of the fluorescence assay was not optimized. The fluorescence-based assay offers a number of advantages over the potentiometric sensor for an automated Biochemical Detector, including speed of measurement, greater stability of reagents and absence of substrate fluidics.

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